

Single Stranded Oligonucleotides, Probes, Primers and Method for Detecting Spirochetes

This invention concerns the techniques for the detection and/or amplification and sequencing, using probes or oligonucleotide primers, and the application of these probes or primers to detect or identify bacteria in the order of *Spirochaetales* (spirochetes).

In certain diagnostic tests, in particular during the search for a human or animal infection in the nervous system or a search after an arthropod bite, for example, it is often necessary to obtain a fast response concerning the presence of bacteria, and more specifically spirochetes, in a sample. However, the techniques currently developed, such as direct examination after Gram coloration or culture often fail due to the lack of Gram colour and the lack of growth in the culture medium.

In the case of spirochetes, forming a family of bacteria including the species *Leptospira*, *Borrelia*, *Treponema* and *Serpulina*, responsible for infectious diseases, such as meningoencephalitis, a culture is not possible. Therefore, the identification of spirochetes has still not been solved.

Given the increase in infectious diseases over the last twenty years and the dramatic consequences of these infectious diseases, it is necessary to develop a fast and specific method to detect infectious pathogenics such as spirochetes.

A possible solution, able to palliate the impossibility of the culture of bacteria, lies in the use of technologies related to nucleic acids and genetic material, in particular PCR (Polymerase Chain Reaction) methods, in order to determine whether a gene, a part of a gene or a nucleotide sequence is present in a living organism, a cell extract from this organism or a sample. Given that any gene or part of a gene is characterised by a specific sequence of nucleotide bases, it is therefore possible to directly search for the presence of all or part of the said specific sequence within a sample containing a mixture of polynucleotides.

Different types of methods for the detection of nucleic acids are described in the literature. These methods are based on the properties of purine-pyrimidine pairing of complementary stands of nucleic acids in the DNA-DNA and RNA-RNA duplex. This pairing process is carried out by the establishment of hydrogen bonds between adenine-thymine (A-T) and guanine-cytosine (G-C) bases of the double strand DNA. Pairs of adenine-uracile (A-U) bases may also form by hydrogen bond in the DNA-RNA or RNA-RNA duplex. The pairing of strands of nucleic acid to determine whether a given molecule of nucleic acid is present or absent is commonly called "hybridation of nucleic acids" or simply "hybridation".

An example of the PCR method includes the determination of a sequence on the base of RNAr 16S. However, this method has limits due to potential problems of contamination that hinder the diagnosis.

To make up for this disadvantage, new genetic markers have been found for the specific detection of bacteria belonging to the order of spirochetes in any sample, without a preliminary stage of bacteria culture. These new markers or oligonucleotides are the object of the invention.

The determination of these new markers relies on the use of specifically defined sequences in the gene *rhoB* of spirochetes coding for the β sub-unit in bacterial RNA polymerase. In fact, zones that vary according to the bacteria family but that appear preserved among the order of spirochetes are found on DNA coding for the β sub-unit of bacterial RNA polymerase. Therefore, this

bacteria family can be distinguished from other bacteria families. The observation that, in the said preserved zones, there are minor variations of sequences between certain species of spirochetes was used to develop these markers specific to the order of spirochetes.

According to Lazcano et al. [J. Mol. Evol. (1988) 27:365-376], the RNA polymerase are divided into two groups according to their origin, one formed by viral RNA- or DNA-dependent RNA polymerase and the other formed by DNA-dependent RNA polymerase of eucaryote or procaryote origin (archaebacteria and eubacteria). The DNA-dependant eubacteria RNA polymerase are characterised by a simple and preserved multimeric constitution called "core enzyme", represented by $\alpha\beta\beta'$ or "holoenzyme" represented by $\alpha\beta\beta'\sigma$ [Yura and Ishihama, Ann. Rev. Genet. (1979) 13 : 59-97].

A great many studies have revealed the functional role, within the multimeric enzyme complex, of the β sub-unit of eubacteria RNA polymerase. Archaeobacteria and eucaryote RNA polymerase present a more complex structure that may include ten or even thirty sub-units [Pühler et al., Proc. Natl. Acad. Sci. USA (1989) 86 : 4569-4573].

The genes that code for the different $\alpha\beta\beta'\sigma$ sub-units of DNA-dependent RNA polymerase in eubacteria, respectively genes *rpoA*, *rpoB*, *rpoC* and *rpoD*, are classified in different groups including genes coding for the proteins that constitute ribosome sub-units or for the enzymes involved in the duplication and repair of the genome [Yura and Ishihama, Ann. Rev. Genet. (1979) 13 : 59-97]. Certain authors have demonstrated that the nucleic sequences of genes *rpoB* and *rpoC* may be used to build phylogenetic trees [Rowland et al., Biochem. Soc. Trans. (1992) 21 : 40s] to separate the different branches and sub-branches among the kingdom of the living.

Before describing the invention in more detail, the different terms used in the description and claims are defined below:

- "nucleic acid extracted from bacteria" refers to either the total nucleic acid or the genomic DNA or the messenger RNA or the DNA obtained from the reverse transcription of the messenger RNA;
- "nucleotide fragment" or "oligonucleotide" are two synonymous terms indicating a sequence of DNA or RNA nucleotide motifs characterised by an informational sequence of natural (or modified) nucleic acids that are likely to hybrid, such as natural nucleic acids, with a complementary or a roughly complementary nucleotide fragment, in predetermined conditions of strict stringence. The sequence may contain nucleotide motifs with a different structure from that of natural nucleic acids. A nucleotide fragment (or oligonucleotide) may, for example, contain up to 100 nucleotide motifs. It generally contains at least 10, and in particular at least 12 nucleotide motifs and may be obtained from a natural nucleic acid molecule and/or by genetic recombination and/or by chemical synthesis;
- "a nucleotide motif" is derived from a monomer that may be a natural nucleotide of nucleic acid where the constitutive elements are a sugar, a phosphate group and a nitrogen base chosen from among adenine, guanine, uracile, cytosine and thymine; or the monomer is a nucleotide modified in at least one of the former three constitutive elements. By way of example, the modification may occur in the bases, with modified bases such as inosine that can hybrid with any base A, T, U, C or G, methyl-5-deoxycytidine, deoxyuridine, dimethylamino-5-deoxyuridine or any other modified base able

to hybrid, or in the sugar, for example, the replacement of at least one deoxyribose by a polyamide [PE Nielsen et al., Science, (1991) 254 : 1497-1500] or in the phosphate group, for example by replacement by chosen esters, in particular among diphosphates, alkyl and arylphosphonates and phosphorothioates;

- "informational sequence" refers to any ordered sequence of nucleotide motifs, where the chemical nature and the order in a reference direction form information similar to that provided by a sequence of natural nucleic acids;
- "hybridation" refers to the process where, in appropriate conditions, two nucleotide fragments with sufficiently complementary sequences are able to associate by stable and specific hydrogen bonds, to form a double strand. The conditions for hybridation are determined by the "stringence", that is, the strictness of the operating conditions. The greater the stringence, the more specific the hybridation. The stringence is mainly a function of the composition in bases of a probe/target duplex, as well as by the degree of mis-matching between two nucleic acids. The stringence may also be a function of the parameters of the hybridation reaction, such as the concentration and type of ionic species found in the hybridation solution, the type and concentration of denaturing agents and/or the hybridation temperature. The stringence of the conditions in which the hybridation reaction has to be carried out mainly depends on the probes used. All of this data is well known and the appropriate conditions may eventually be determined in each case by routine experiments. In general, depending on the length of the probes used, the temperature for the hybridation reaction is approximately between 20 and 65 °C, in particular between 35 and 65 °C in a saline solution at a concentration of about 0.8 to 1 M.
- a "probe" is a nucleotide fragment including, for example, between 10 and 100 nucleotide motifs, in particular between 12 and 35 nucleotide motifs, with a hybridation specificity in determined conditions to form a hybridation complex with a nucleic acid with, in the present case, a nucleotide sequence included in a messenger RNA or in a DNA obtained by reverse transcription of the said messenger RNA, a transcription product. A probe may be used for diagnostic purposes (in particular capture or detection probes) or for therapeutic purposes;
- a "capture probe" is immobilised or can be immobilised on a solid support by any appropriate means, for example, by covalence, by adsorption, or by direct synthesis on a solid. Examples of supports include microtitration plates and DNA chips;
- a "detection probe" may be marked by means of a marking agent chosen for example from among the radio-active isotopes, the enzymes, in particular enzymes able to act on a chromogenic, fluorogenic or luminescent substrate (in particular a peroxydase or an alkaline phosphatase), chromophoric chemical compounds, chromogenic, fluorogenic or luminescent compounds, analogues of nucleotide bases and ligands such as biotine;
- a "species probe" is a probe that is able to identify the species of bacteria;
- a "genus probe" is a probe that is able to identify the genus of a bacteria;
- a "primer" is a probe including, for example, between 10 and 100 nucleotide motifs and with a hybridation specificity in determined conditions for the initiation of an enzyme polymerisation, for example, in an amplification

technique such as PCR, in a sequencing procedure, in a transcription method, etc.

The first purpose of the present invention is a single stranded oligonucleotide chosen from among the oligonucleotides with a sequence of at least 12 consecutive nucleotide motifs included in one of the sequences SEQ ID N° 1 to SEQ ID N° 4 described in the list of sequences at the end of the description and among the complementary oligonucleotides of these oligonucleotides.

In each of the sequences SEQ ID N° 1 to 4, the nucleotide "N" mentioned in the list of sequences at the end of the description represents an identical or different nucleotide in each of the 3 positions where it appears and may represent inosine or an equimolar mixture of 4 different nucleotides chosen from among A, T, C and G or, should the occasion arise, A, U, C and G.

When "N" represents a said equimolar mixture of nucleotides at a given position, this means that the nucleotide at the said position indifferently represents A, T, C or G (or respectively, should the occasion arise, A, U, C or G) and that the oligonucleotide according to the invention more exactly consists of an equimolar mixture of 4 groups of oligonucleotides in each the said groups "N" has a different meaning at the said given position and respectively represents in each of the 4 groups A, T, C and G.

In each of the sequences SEQ ID N° 1 to 4, when in the 3 positions where a nucleotide "N" appears, N represents a said equimolar mixture of 4 nucleotides A, T, C and G (or N should the occasion arise A, U, C and G). The oligonucleotide forming the said sequence therefore represents a mixture of 4^3 (64) oligonucleotides.

As indicated in the definitions, the oligonucleotide according to the invention may be in the form of an oligodeoxyribonucleotide (DNA) or an oligoribonucleotide (RNA) where, in this case, "T" is replaced by "U".

In particular, an oligonucleotide according to the present invention has at least 12 motifs as described above and at not more than 50 motifs. Specifically, according to the present invention, an oligonucleotide has from 12 to 35 motifs.

A preferred oligonucleotide has a sequence chosen from among the sequences SEQ ID N° 1 to 4, or the complementary sequences.

Sequence SEQ ID N° 1, the sequence from the primer called primer N° 1, has 20 nucleic acids. Sequence SEQ ID N° 2, the sequence from the primer called primer N° 2, has 21 nucleic acids. Sequence SEQ ID N° 3, the sequence from the primer called primer N° 3, has 20 nucleic acids. Sequence SEQ ID N° 4, the sequence from the primer called primer N° 4, has 17 nucleic acids.

In the nomenclature referring to gene *rpoB* in *E. COLI*, the first nucleotides in sequences SEQ ID N° 1 to 4 correspond to the following positions:

- SEQ ID N° 1 : 1 730,
- SEQ ID N° 2 : 2 900,
- SEQ ID N° 3 : 3 700,
- SEQ ID N° 4 : 3 850.

The inventors discovered and revealed that the said sequences SEQ ID N° 1 to 4, as defined above, are not only consensual between the spirochetes, but that they are also specific to the family of spirochetes and enclose a fragment of gene *rpoB* including a very variable zone where the sequence is specific to the species and the genus serovar in a species of *spirochete*.

In fact, variable nucleotides in the complementary target sequences are found in the positions corresponding to those of nucleotides "N" in the sequences SEQ ID

N° 1 to 4 as a function of the species of bacteria considered. However, the other nucleotides are preserved in all of the species of bacteria in the spirochete family. Since "N" represents inosine that may hybrid with any base, or an equimolar mixture of 4 bases A, T, C and G, an oligonucleotide or a mixture of oligonucleotides according to the invention always includes an oligonucleotide that can hybrid with a complementary target sequence of a spirochete bacteria.

In addition, since the sequences SEQ ID N° 1 to 4 enclose variable zones where the sequences are specific for each species of bacteria in the spirochete family, sequences SEQ ID N° 1 to 4 not only allow for the preparation of specific probes for the family of spirochetes, but also the detection and identification of the species of the said bacteria by PCR amplification by using the said sequences as primers.

In a mode of preparation of oligonucleotides according to the invention, "N" represents an inosine in all the positions.

In another mode of preparation, the oligonucleotide according to the invention represents a mixture of oligonucleotides (in particular 4^P oligonucleotides) comprising sequences included in one of the sequences SEQ ID N° 1 to 4, in which all of the nucleotides A, T, C and G are represented in each of the said positions where "N" appears (in particular p positions, p representing a whole number).

Sequences SEQ ID N° 1 to 4 may be prepared by chemical synthesis using familiar techniques described, for example, in the article by Itakura K. et al. [(1984) Annu. Rev. Biochem. 53 : 323].

A first application of an oligonucleotide from the invention is its use as a probe for the detection, in a biological sample, of bacteria belonging to the order of spirochetes that include a nucleotide sequence with at least 12 consecutive nucleotide motifs included in one of the sequences SEQ ID N° 1 to 4, and their complementary sequences. In the rest of the description, such a probe will be called a "genus probe".

According to the invention, the probes may be used for diagnostic purposes, in the search to determine whether a target nucleic acid is present in a sample, according to all known hybridation techniques and in particular the so-called "DOT-BLOT" techniques involving the deposit on a filter [Maniatis et al. (1982) Molecular Cloning, Cold Spring Harbor], the so-called "SOUTHERN BLOT" techniques involving DNA transfer [Southern, E.M., J. Mol. Biol. (1975) 98 : 503], the so-called "NORTHERN BLOT" techniques involving RNA transfer, or the so-called "sandwich" techniques [Dunn A.R., Hassel J.A. (1977) Cell 12 : 23]. In particular, the "sandwich" technique is used with a capture probe and/or a detection probe, the said probes are able to hybrid with two different regions of target nucleic acid, and at least one of the said probes (generally the detection probe) is able to hybrid with a region in the target that is specific to the species or group of species searched for, considering that the capture probe and the detection probe have to have at least partially different nucleotide sequences.

The nucleic acid to detect (target) may be DNA or RNA (one of them possibly obtained after PCR amplification). In the case of the detection of a double strand nucleic acid target, it is advisable to proceed with the denaturation of the latter before the use of the detection procedure. The target nucleic acid may be obtained by extraction according to the known methods for nucleic acids from a test sample. The denaturation of a double strand nucleic acid may be carried out by the familiar methods of chemical, physical or enzyme denaturation, and in particular by heating at an appropriate temperature above 80 °C.

To use the aforementioned hybridization techniques, and in particular the "sandwich" techniques, a probe from the invention, called a capture probe, is immobilised on a solid support, and another probe from the invention, called a detection probe, is marked with an agent.

The examples of support and marker agent are defined above.

Another purpose of the invention is a method to determine whether at least one spirochete is present in a sample containing or likely to contain nucleic acids from at least one such bacteria, including the steps consisting in putting in contact the said sample with at least one genus probe from the invention, then to determine in a known manner whether a hybridation complex has formed between the said probe and the nucleic acid from the sample.

Examples of detection of the formation or lack of formation of a hybridation complex between the said probe and the nucleic acid include the techniques described above, that is, the "DOT-BLOT", "SOUTHERN-BLOT" and "sandwich" techniques.

In one specific use of this procedure to determine whether or not a species or a group of species of spirochete are present, a genus probe from the invention and a species probe from the invention are used. Of course, the said genus and species probes are able to hybrid with the non-overlapping regions of a nucleic acid corresponding to the gene *rpoB* of spirochetes.

In an advantageous way, the genus probe is immobilised on a solid support and the species probe is marked with a marking agent.

The present invention also involves the application of the procedure from the invention to determine the presence of a determined spirochete species.

In fact, the results of the research, revealing alignments of sequences preserved in spirochete species, according to the CLUSTAL method of alignment [Higgins D.G. & Sharp P.M. (1989) *Gene* 73 : 237-244], the said sequences have 1170 bases located between positions 1730 and 2900 of gene *rpoB* by referring to the numbering of *Escherichia coli* gene *rpoB* ATCC 25290, allow for the detection of at least one bacteria in the order of spirochetes. The use of probes containing zones muted for a specific species (with respect to the reference species, in this case *E. coli*) makes it possible to directly detect such a species. The sandwich hybridation techniques, using a combination of two probes (capture probe and detection probe), use, for example, a combination of a probe specific for the family of spirochetes and a probe specific for the species considered. It is also possible to use a combination of two probes specific for the said species. When they exist, these two probes are complementary for non-overlapping regions of the gene *rpoB*.

Another application of an oligonucleotide in the invention is its use as a nucleotide primer including a single stranded oligonucleotide chosen from among the oligonucleotides with a sequence of at least 12 nucleotide motifs included in one of the sequences SEQ ID N° 1 to 4, that can be used in the synthesis of a nucleic acid in the presence of a polymerase by a known procedure, that is in the methods of amplification using such a synthesis in the presence of a polymerase (PCR, RT-PCR, etc.). In particular, a primer from the invention may be used for the specific reverse transcription of a sequence of messenger RNA of at least one species or at least a group of species of spirochetes to obtain a corresponding complementary sequence of DNA. Such a reverse transcription may be the first stage in the RT-PCR technique, the following stage being the amplification of the complementary DNA obtained by PCR. It is also possible to use primers from the invention for the specific

amplification by chain polymerisation reaction of the DNA sequence of the gene *rpoB* of at least one species or at least a group of species of spirochetes.

In a specific case, since the said primer comprises an oligonucleotide from the invention it also includes the direction or anti-direction sequence of a promotor recognised by a RNA polymerase (for example, promotors T7, T3, SP6 [Studier FW, BA Moffatt (1986) J. Mol. Biol. 189 : 113]. Such primers can be used in the methods to amplify nucleic acid involving a transcription stage, such as, for example, the NASBA or 3SR techniques [Van Gemen B. et al. Abstract MA 1091, 7th International Conference on AIDS (1991), Florence, Italy].

Another purpose of the invention is a nucleotide primer comprising a single stranded oligonucleotide chosen from among the oligonucleotides with a sequence of at least 12 consecutive nucleotide motifs included in one of the sequences SEQ ID N° 1 to SEQ ID N° 4 that can be used for the total or partial sequencing of the gene *rpoB* in any species of spirochete. In particular, the nucleotide primer can be used for the sequencing of an amplified nucleic acid.

In fact, the oligonucleotide primers concerned by the invention allow for the amplification and then the sequencing of the gene *rpoB* in all spirochetes, and the identification of any spirochete by bio-data processing analysis of this sequence and the recognition of new and unknown species of spirochetes. The sequencing involves the acquisition of the total or partial sequence of the gene *rpoB* by a familiar method, absorptive polymerisation using di-deoxynucleotides [Sanger F., Coulson A.R. (1975) J. Mol. Biol. 94 : 441] or multiple hybridations using DNA chips.

Primers N° 1 to N° 3 can be used as 5' primers and primers N° 2 to N° 4 as 3' primers to amplify a fragment of gene *rpoB* enclosed in 5' and 3' by the said primers.

In one mode, a set of two primers is used consisting of two different oligonucleotides chosen from among the oligonucleotides with a sequence of at least 12 nucleotide motifs included in sequences SEQ ID N° 1 to 3 for 5' primer and SEQ ID N° 2 to 4 for 3' primer.

The present invention also includes a method, characterised in that the fragment of gene *rpoB* of the said bacteria is first amplified with primers according to the invention, then the said fragment is put into contact with a probe from the said bacteria according to the invention. Whether or not a hybridation complex is formed between the said probe and the said fragment is determined.

The present invention also involves a method to determine whether at least one bacteria in the order of *spirochaetales* is present in a sample containing or likely to contain nucleic acids from at least one such bacteria, characterised in that the said sample is put into contact with primers according to the invention. Amplification is then carried out and whether or not an amplification product appears is determined.

In one mode, a method to determine whether at least one bacteria from the order of *spirochaetales* is present in a biological sample containing or likely to contain nucleic acids from the said bacteria, is characterised in that the method amplifies and carries out the sequencing of a fragment of gene *rpoB* of the said bacteria in the said sample by means of primers according to the invention and the sequence of the fragment of gene *rpoB* of the gene obtained is compared with the known sequence of a fragment of gene *rpoB* of the said bacteria in a sample if the sequence of the fragment obtained is identical to the known sequence of the fragment of gene *rpoB* of the said bacteria.

The present invention also involves a detection probe, in a biological sample, specific for a species of bacteria belonging to the order of *spirochaetales*, characterised in that it includes a fragment of gene *rpoB* that can be obtained by

amplification by means of primers according to the invention, preferably with one of the sequences SEQ ID N° 1 to 3 as 5' primer and one of the sequences SEQ ID N° 2 to 4 as 3' primer. The said fragment of gene *rpoB* enclosed by the oligonucleotides according to the invention actually corresponds to a very variable zone according to the species of said bacteria belonging to the order of *spirochaetales* and that is specific for each said species.

Finally, the invention also includes a gene therapy probe to treat infections induced by at least one species or a group of species of spirochetes, the said probe including an oligonucleotide as defined above. This gene therapy probe, able to hybrid on the messenger RNA and/or on the genome DNA of the said bacteria, can block the translation and/or transcription and/or duplication phenomena.

The principle of the gene therapy methods is known and is mainly based on the use of a probe corresponding to an anti-direction strand. The formation of a hybrid between the probe and the direction strand is able to perturb at least one of the stages in the decoding of the genetic information. Gene therapy probes can thereby be used as anti-bacteria drugs, to fight infections caused by spirochetes.

The invention will now be described in detail using the following experimental report.

The following description will better be understood using figures 1 and 2 in which:

- figure 1 is a photo of an electrophoresis gel revealing the detection of spirochetes in a mixed bacteria suspension using the probes in the invention; and
- figure 2 is a photo of an electrophoresis gel revealing the specific amplification by PCR of fragments of gene *rpoB* of spirochetes by using the primers in the invention.

The strains of spirochetes used in the following examples, that is *Borrelia burgdorferi*, *Borrelia recurrentis*, *Treponema pallidum*, *Leptospira biflexa* serovar *patoc* (hereafter called *Leptospira biflexa*), *Leptospira interrogans* serovar *icterohaemorrhagiae* (hereafter called *Leptospira icterohaemorrhagiae*) and *Leptospira interrogans* serovar *australis* (hereafter called *Leptospira australis*), have all been obtained from the ATCC collection, except for *Borrelia recurrentis* that is available at the Centre National de référence, Institut Pasteur Paris (France). The ATCC number of *Borrelia burgdorferi* is 35210, that of *Treponema pallidum* is 27087, that of *Leptospira biflexa* is 23582, that of *Leptospira icterohaemorrhagiae* is 43642 and that of *Leptospira australis* is 23605.

The *Borrelia* and *Leptospira* strains were grown at 30 °C on BSKII and EMJH media, respectively [Barbour A.G. (1984) Yale J. Biol. Med. 57 : 521]. Since *T. pallidum* can not be grown *in vitro*, this pathogenic bacteria was propagated by injection in the testicles of a rabbit.

The other strains of bacteria used, *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus salivarius* and *Pseudomonas aeruginosa* were clinically isolated from patients hospitalised in Marseille.

EXAMPLE 1: Specific detection of spirochetes using probes from the invention

This experiment was carried out with the following spirochete strains: *Borrelia burgdorferi*, *Treponema pallidum*, *Leptospira biflexa*, *Leptospira icterohaemorrhagiae*, *Leptospira australis* and *Borrelia recurrentis*, as well as with *Staphylococcus aureus*.

A mixed bacteria suspension was prepared by mixing a spirochete DNA with a extract of *Staphylococcus aureus*.

A PCR method was carried out using the QIAamp tissue kit (Qiagen) and the Gibco Polymerase Taq (Gibco BRL, USA). After a first stage of determination (94 °C for 2 minutes), a cycle of 3 stages consisting of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 minute was repeated 35 times. The PCR programme was terminated. The resulting amplicons were purified and sequenced as indicated above.

The results are indicated in figure 1, in which:

band 1 corresponds to molecular mass markers (Boehringer),

band 2 corresponds to *S. aureus* alone,

band 3 corresponds to *B. burgdorferi* + *S. aureus*,

band 4 corresponds to *B. recurrentis* + *S. aureus*,

band 5 corresponds to *T. pallidum* + *S. aureus*,

band 6 corresponds to *L. biflexa* + *S. aureus*,

band 7 corresponds to *L. australis* + *S. aureus*,

band 8 corresponds to *L. icterohaemorrhagiae* + *S. aureus*.

The following primers were used:

panel A: RNAr primers 16S FDI and RD3 [Weisburg et al. J. Bacteriol. (1991) 173 : 697-703].

panel B: the primers 1730D and 2900R from the invention.

The primer 1730D in sequence SEQ ID N° 1 (5'CTTGGNCCNGGNGGACTTTC3') in which "N" is inosine and the primer 2900R has the sequence SEQ ID N° 2 (5'AGAAATNAANATNGCATCCTC3') in which "N" is inosine.

The results show that the primers in the invention were not able to amplify the DNA of *S. aureus* alone, and therefore detect *S. aureus*, but were able to amplify that of the spirochetes, and thereby detect it, even in the presence of *S. aureus* (panel B), as opposed to primers 16S that were able to amplify the DNA only in *S. aureus* (panel A).

EXAMPLE 2: Specific amplification of fragments of gene *rpoB* using the primers in the invention

This experiment was carried out with the following spirochete strains: *Borrelia burgdorferi*, *Treponema pallidum*, *Leptospira biflexa*, *Leptospira icterohaemorrhagiae*, *Leptospira australis* and *Borrelia recurrentis*, as well as with the other non-spirochete strains *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus salivarius* and *Pseudomonas aeruginosa*.

The sequence of the gene *rpoB* in *Treponema pallidum* used is that described by Weinstock et al. [(1998) The genome of *Treponema pallidum*: new light on the agent of syphilis. FEM Microbiol. Rev. 22 : 323-332].

The sequence of gene *rpoB* in *Borrelia burgdorferi* used is that described by Alekshun et al. [(1997) Molecular cloning and characterisation of *Borrelia burgdorferi* *rpoB*. Gene 186 : 227-235].

The DNA sequence of the gene *rpoB* of *Leptospira biflexa*, *Leptospira icterohaemorrhagiae*, *Leptospira australis* and *Borrelia recurrentis* were obtained by PCR in the following way:

The genome DNA of *Leptospira biflexa* was extracted according to the standard procedures with phenol/chloroform [Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbour, NY]. In this first stage of the

experiment, amplifications by PCR were carried out with primers SEB1 (5'-AAC CAG TTC CGC GTT GGC CTG G-3') and SEB2 (5'-CCT GAA CAA CAC GCT CGG A-3') (SEB for *Staphylococcus aureus*, *Escherichia coli* and *Borrelia subtilis*) and the polymerase DBA *Taq* by Eurogentec (Seraing, Belgium) by using 5 µl of DNA for a final volume of 50 µl. After a first stage of denaturation (95 °C for 1.5 min), a cycle was repeated 35 times consisting of 3 stages of 95 °C for 20 s, 50 °C for 30 s and 72 °C for 1 min. The PCR programme was completed with a single stage of extension of 3 min at 72 °C (Peltier PTC 200 heat cycle model, MJ Research, Watertown, MA, USA).

The amplicons obtained by electrophoresis were then separated on 1 % agarose gel and they were visualised by coloration with ethidium bromide.

The sequencing of the gene was then carried out by purification of the samples with the PCR QIAquick purification kit (Qiagen, Hilden, Germany) and they were allowed to react with the dRhodamine Terminator Cycle Sequencing Ready Reaction buffer (DNA sequencing kit, Perkin-Elmer). Finally, the electrophoresis of the reactive products was carried out with the automatic Applied Biosystems DNA sequencer model ABI 310 (Perkin-Elmer).

The sequence of the 5' end of gene *rpoB* was obtained by using a Universal Genome Walker™ kit (Clontech, Palo Alto, CA, USA) in the following manner. Five groups of genome DNA fragments called Genome Walker "banks" were built by using *DraI*, *EcoRV*, *PvuII*, *ScaI* and *StuI* restriction enzymes. These banks were used for the prior sequencing of the genome DNA by using a specific primer for the gene whose sequence corresponds to the region as close as possible to the known 5' end of the gene and an adaptation primer supplied by the kit.

The amplified fragments were purified and a source of DNA was used for a second enclosed PCR. The amplicons obtained were then treated as described above for the automatic sequencing.

Gene *rpoB* from the *Leptospira biflexa* obtained has the sequence SEQ ID N° 5 and has 3876 nucleic acids. Gene *rpoB* from the *Leptospira icterohaemorrhagiae* obtained has a sequence SEQ ID N° 6 and has 914 nucleic acids. Gene *rpoB* from the *Leptospira australis* obtained has the sequence SEQ ID N° 7 and has 949 nucleic acids. Finally, gene *rpoB* from the *Borrelia recurrentis* obtained has the sequence SEQ ID N° 8 and has 800 nucleic acids.

The DNA sequences of the gene *rpoB* from *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus salivarius* and *Pseudomonas aeruginosa* are described in the literature (Gene Bank).

The fragments of gene *rpoB* to amplify consisted of 949 pb.

The amplifications of the fragments of gene *rpoB* by PCR were carried out as in example 3, except that each species was used separately.

The results are indicated in figure 2 where:

- band 1 corresponds to the molecular mass markers (Boehringer),
- band 2 corresponds to *B. burgdorferi*,
- band 3 corresponds to *B. recurrentis*,
- band 4 corresponds to *T. pallidum*,
- band 5 corresponds to *L. biflexa*,
- band 6 corresponds to *L. australis*,
- band 7 corresponds to *L. icterohaemorrhagiae*,
- band 8 corresponds to *E. coli*,
- band 9 corresponds to *S. aureus*,
- band 10 corresponds to *Str. salivarius*,

band 11 corresponds to *Ps. aeruginosa*,

band 12 corresponds to a negative reference without DNA.

The following primers were used:

panel A: primer 1730D and primer 2900R from the invention,

panel B: primer 1730D and primer 3800R from the invention and

panel C: RNAr primers 16S RD1 and FD3.

Primer 3800R has the sequence SEQ ID N° 4 (5'GCTTCNAGNGCCCANAC3') in which "N" is an inosine.

The results show that the primers in the invention only allowed for the amplification of the DNA fragments of spirochetes (panels A and B) while primers 16S amplified everything (panel C).

The primer SEQ IS N° 3 (5'GGGTGNATTTTNTCATCHAC3') may also be used as a primer. Primers N° 1 and 3 according to the invention allowed for the amplification and then sequencing of the amplified product, the detection and identification of the bacteria *Borrelia duttonii* in which an African tick collected in ZAIRE of the species *ornithodoros moubata* and the total blood of a Zaire patient presenting a recurrent fever.